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Effect of Processing on Recovery and Variability Associated with Immunochemical Analytical Methods for Multiple Allergens in a Single Matrix: Dark Chocolate

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S *Supporting Information*

ABSTRACT: Immunodetection of allergens in dark chocolate is complicated by interference from the chocolate components. The objectives of this study were to establish reference materials for detecting multiple allergens in dark chocolate and to determine the accuracy and precision of allergen detection by enzyme-linked immunosorbent assay (ELISA) before and after chocolate processing. Defatted peanut flour, whole egg powder, and spray-dried milk were added to melted chocolate at seven incurred levels and tempered for 4 h. Allergen concentrations were measured using commercial ELISA kits. Tempering decreased the detection of casein and β-lactoglobulin (BLG), but had no significant effect on the detection of peanut and egg. Total coefficients of variation were higher in tempered than untempered chocolate for casein and BLG, but total and analytical CVs were comparable for peanut and egg. These findings indicate that processing has a greater effect on recovery and variability of casein and BLG than peanut and egg detection in a dark chocolate matrix.

KEYWORDS: *allergen detection, chocolate matrix, ELISA methods, food allergen, incurred reference material, thermal processing*

INTRODUCTION

The prevalence of peanut, egg, and milk allergies is increasing in the United States (U.S.), especially in children. Among regulated food allergens, these three account for the majority of symptomatic responses[1] and are also responsible for the majority of allergen-related safety recalls by the U.S. Food and Drug Administration.[2] Many factors, including geography, dietary habits, and genetics, may predispose susceptible individuals to food allergy. Exposure to the allergenic food can be limited by following a strict avoidance diet, but allergic consumers are vulnerable when foods contain ambiguous ingredient labels or undeclared allergens. Because trace amounts of allergenic proteins in processed foods can cause life-threatening reactions in some sensitized patients, accurate and sensitive immunologic methods are required for allergen quantitation.

Processed foods containing allergenic material are consumed more often than pure allergenic foods and are more frequent causative agents of allergic responses in sensitive individuals.[3−5] During the manufacturing process, allergenic proteins undergo various physical and chemical modifications such as unfolding, aggregation, hydrolysis, or covalent modification, but most retain their allergenic potential.[6−9] The impact of processing on allergen detection by immunoassays was evaluated by analyzing oil- or dry-roasted peanuts; autoclaved peanuts; heated, boiled, and autoclaved egg powder; and heated milk.[10−17] Fewer immunochemical studies have evaluated allergenic food residues that are incorporated in a food matrix and processed using standard manufacturing processes.[18−23] Each food matrix and processing condition varies, and it therefore may not be feasible to determine the effect of processing on all allergens in all processed foods.

In addition to the effects of processing on the allergenic protein, the components of the food matrix may hamper critical procedural steps of immunoassays, such as allergen extraction or interference with antibody recognition of the targeted protein. Food matrix components also can change the pH, solubility, and chemical structure of the allergenic proteins following processing or may directly interfere with the enzymatic activity of the enzyme−conjugate kit reagent. Matrix components may also cross-react with antibodies of the ELISA kit, causing false-positive results. Dark chocolate is one of the more complicated food matrices. In addition to the interfering components of chocolate, such as tannins and other polyphenols, the physical texture and fat content cause difficulties in preparing homogeneous samples and antibody detection.[24−27]

ELISAs are more widely used than other available detection methods because they are easy to use and are sensitive and

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specific for allergenic protein detection. The use of commercial ELISA kits for the quantification of food allergens, however, is associated with inherent uncertainty. The kits differ in accuracy and precision because the component antibodies often differ in sensitivity and specificity. In addition, the kits use different extraction buffers and procedures, as well as different serial calibrators and data reduction methods. All of these critical parameters contribute to differences in the performance of the various test kits for detecting a particular allergen.[28−32] The use of incurred samples can help to identify some of the inherent problems of allergen ELISAs and to improve the reliability of allergen detection methods. Here we examined the feasibility of using an incurred model food containing multiple allergens in a single matrix for this purpose.

Defatted peanut flour, spray-dried whole egg powder, and nonfat milk powder were added to dark chocolate at seven incurred levels, and the chocolate was then tempered according to industry standards. Sample aliquots from each incurred level were removed prior to tempering. ELISA kits from five commercial vendors were used for allergen quantitation using the untempered and tempered samples. The objectives of this study were to (1) develop incurred reference materials in a dark chocolate matrix, (2) determine if multiple allergens can be incorporated simultaneously in the same dark chocolate matrix, and (3) evaluate the effect of processing on the accuracy and precision of peanut, egg, and milk ELISA detection methods using this incurred matrix. In addition, we examined the critical parameters associated with detection variability when using immunochemical analytical methods for the quantitation of test allergens in dark chocolate.

MATERIALS AND METHODS

Food Samples. Tempered and untempered chocolate samples were studied. All of the controls and incurred samples were produced according to industry standards at the U.S. Food and Drug Administration, Institute for Food Safety and Health (Bedford Park, IL, USA).

Incurred Dark Chocolate Ingredients. The ingredients were Scharffen Berger semisweet 62% cacao chocolate (dark chocolate), produced on a peanut-, egg-, and milk-free dedicated line (Scharffen Berger, Berkeley, CA, USA); nonfat dry milk-NIST SRM 1549 (National Institute of Standards and Technology, Gaithersburg, MD, USA); spray-dried whole egg powder-NIST RM 8445 (National Institute of Standards and Technology); and light-roasted peanut flour, 12% fat light roast, product 521271, lot 109FA (Golden Peanut Co., Alpharetta, GA, USA).

Preparation of Untempered Chocolate Samples. Dark chocolate was ground to a fine powder using an IKA analytical mill (Wilmington, NC, USA) with pulverized dry ice added to the grinding chamber. The fine powder was separated from the larger particles by straining. The 500 ppm (1 ppm = 1 μg/g) allergen-containing sample was made by mixing the required amount of dark chocolate powder, dry milk, egg powder, and peanut flour. This sample was then used to prepare 100, 25, 10, 5, and 2.5 ppm allergen-containing samples.

Preparation of Tempered Chocolate Samples. The dark chocolate was chopped into workable pieces. For processing, a bowl containing the chopped chocolate was placed into a tempering machine (preheated to 42 °C for approximately 15 min) to melt the chocolate for 30 min while being mixed with a Teflon scraper. For the 500 ppm incurred sample, the required amounts of dry milk, egg powder, and peanut flour were added to the chocolate and mixed for 30 min in the tempering machine. Samples containing 100, 25, 10, 5, and 2.5 ppm of allergens were made by mixing the required amount of the 500 ppm incurred sample with melted chocolate. The temperature was then increased to 46 °C, and the mixing procedure was continued for 4 h to ensure homogeneity of the chocolate samples. The melted chocolate was then allowed to harden at room temperature for 2 h and placed in the refrigerator for another 2 h to ensure that the chocolate was brittle enough to grind in a food processor.

Test Kits. The five commercial test kits used in this study were (1) RIDASCREEN FAST peanut, egg, and casein from R-Biopharm (RB, Washington, MO, USA); (2) Veratox peanut, egg, and total milk allergen quantitative test kits from Neogen (NE) Corp. (Lansing, MI, USA); (3) Morinaga (MO) peanut, egg, and milk (casein and BLG) protein ELISA kits (Crystal Chem, Downers Grove, IL, USA); (4) Tepnel (TE) BIOKITS peanut, egg, casein, and BLG assay kits (Neogen Corp.); and (5) ELISA Systems (ES) peanut, egg, casein, and BLG residue kits (BioMerieux, Durham, NC, USA).

Analytical Methods. The characteristics of the commercial kits evaluated in this study are listed in Supplemental Table 1 of the Supporting Information. Allergen extraction and ELISA procedures were performed by following the manufacturers' instructions. The RIDASCREEN BLG kit was not used for this study due to matrix effects with the chocolate samples, as indicated in the kit instructions. Before extraction, both the processed and unprocessed samples were melted at 60 °C for 30 min. The data were analyzed using a Spectramax M5 ELISA plate reader equipped with Softmax Pro 5.3 software (Molecular Devices Corp., Sunnyvale, CA, USA). For quantitation, standard curves were created using the manufacturer's recommended curve fit, or by using a four-parameter logistic calibration curve if none was recommended.

Experimental Design. The experimental design of this study was similar to that described previously for the detection of peanut proteins in foods.[24] A balanced nested design was used to measure peanut, egg, and milk (casein and BLG) proteins using ELISA kits from five commercial vendors. Four samples of untempered and tempered dark chocolate at each incurred level were extracted according to the kit instructions. For each sample, four aliquots were used to quantitate the allergen concentration (16 total aliquots per incurred level). A total of 224 analyses were performed using each kit for one allergen in each of the two chocolate preparations (untempered, tempered; 4 samples × 4 aliquots × 7 incurred concentrations × 2 chocolate preparations). A total of 3360 analyses were performed for peanut, egg, and casein (224 analyses × 5 commercial kits × 3 allergens) and 672 analyses for BLG (224 analyses × 3 commercial kits).

Statistical Analysis. Each kit for each allergen was evaluated for accuracy and precision. Accuracy was defined as the deviation of the mean measured allergen value from the true (incurred) value and is expressed as percent recovery (measured value/incurred value × 100). The relationship between measured and incurred values was determined by regression analysis and is expressed graphically as measured versus incurred protein values. Measured values refer to allergen levels quantitated following the instructions of each test kit, and incurred protein values are calculated on the basis of the protein content of each of the allergen reference materials used for chocolate preparation. Variance (standard deviation squared, SD^2), as a measure of precision, was determined using the Proc Nested procedure in SAS.[33] The total variance was partitioned into sampling variance (defined

as the concentration differences among the four samples) and analytical variance (defined as the concentration differences among the four aliquots of each sample). The sampling and analytical CVs [CV% = 100(sample/analytical SD of measured value/mean measured value)] were also calculated for each test kit and allergen as an additional measure of precision.

RESULTS AND DISCUSSION

Quantitation of Peanut, Egg, and Milk Allergens in Untempered Dark Chocolate. Because chocolate is a difficult matrix for food allergen detection, a major goal of this study was to develop incurred standards for detecting multiple allergens in a dark chocolate matrix.[34] The data obtained from the assays were analyzed on the basis of the calculated protein level (Table 1) of each allergen at each incurred level.[35,36] The mean measured concentrations and percentage of recovery (accuracy) for each test kit at each concentration level are shown in Supplemental Table 2 of the Supporting Information for peanut, egg, casein, and BLG, respectively. Quantitated levels of food allergens in unprocessed dark chocolate depended on the kit and the allergen being detected.

Table 1. Calculated Peanut, Egg, and Milk Protein Content at Indicated Incurred Levels

	content (ppm) at incurred level of peanut flour[a], spray-dried whole egg,[b] and nonfat dry milk[c]						
	0 ppm	2.5 ppm	5 ppm	10 ppm	25 ppm	100 ppm	500 ppm
peanut	0	1.26	2.52	5.04	12.6	50.4	252
egg	0	1.2	2.4	4.8	12	48	240
casein	0	0.72	1.44	2.88	7.2	29	144
BLG	0	0.09	0.18	0.36	0.9	4	18

[a]Partially defatted light-roast peanut flour. Protein: N × 5.46 = 50.39% (N = nitrogen). [b]NIST 8445. Protein: N × 6.25 = 48%. [c]NIST 1549. Protein: 36%.[35] Estimated protein content assuming casein is 80% and BLG is 10% of total milk protein.[36]

Peanut. The ES, MO, TE, and NE kits underestimated the incurred peanut protein at all levels in the untempered samples (Supplemental Table 2A of the Supporting Information). Mean recoveries across all incurred levels for these kits were 1.8% (ES), 17.1% (MO), 11.8% (TE), and 35.1% (NE), shown graphically in Figure 1A. The RB kit accurately measured peanut protein at all incurred levels, with minimal background at the zero incurred level. Recovery averaged across all levels for the RB kit was 92.6%. On the basis of recoveries at the lowest incurred levels (1.26 and 2.52 ppm), the MO, ES, and TE kits were not able to detect peanut protein in these chocolate samples at the limits of quantitation (LOQs) claimed by the manufacturer, increasing the possibility that the MO, ES, and TE kits provide false-negative results at lower incurred peanut protein levels.

Egg. The NE and RB kits overestimated egg protein content at all incurred levels, with mean recoveries of 253.1 and 264.1%, respectively, in untempered dark chocolate (Figure 1B). Using the MO kit, the measured egg protein levels were similar to incurred levels of egg protein, with a mean recovery of 110.4%. Egg protein recoveries using the ES and TE kits were similar at all incurred levels, with mean recoveries of 78.5 and 77.3%, respectively. All of the kits detected egg at the manufacturers'

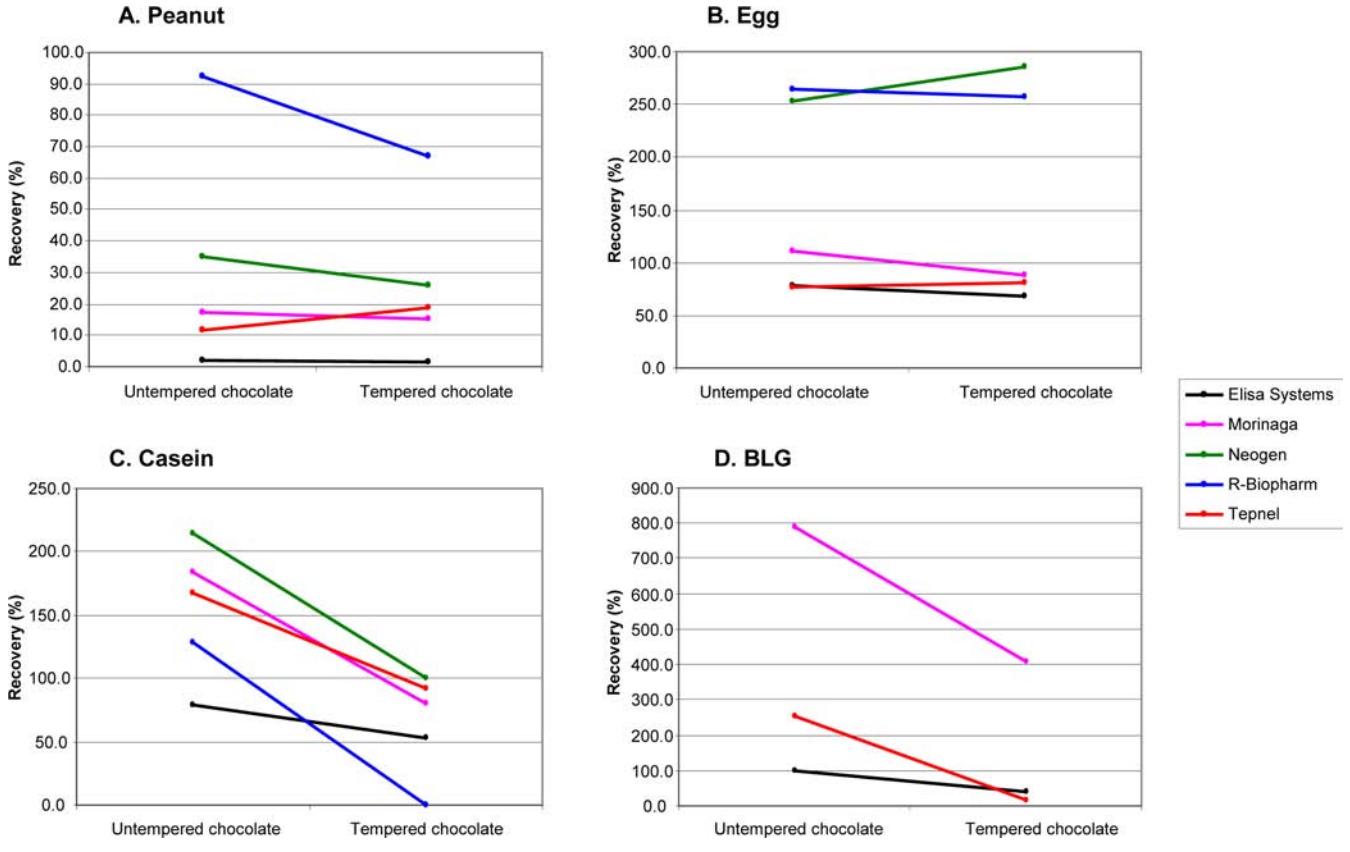

Figure 1. Mean percent recovery of all incurred levels in untempered and tempered dark chocolate for (A) peanut, (B) egg, (C) casein, and (D) BLG (incurred level used to calculate recovery, although the effect of tempering on allergen concentration is unknown).

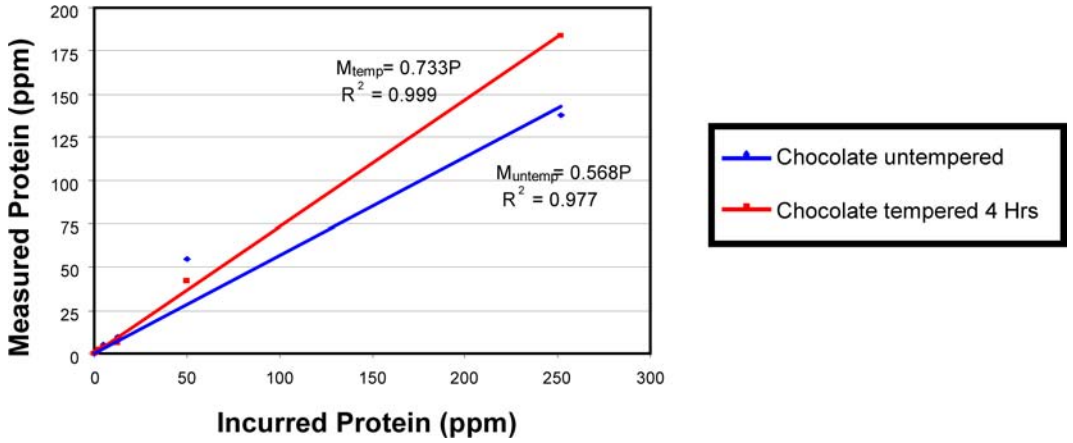

Figure 2. Correlation between measured protein (M) and incurred protein concentration (P) as determined by linear regression analysis for peanut allergen in untempered and tempered dark chocolate using the R-Biopharm (RB) peanut protein kit.

claimed LOQs in untempered chocolate standards, although the ES kit underestimated the egg protein content at the two lowest incurred levels.

Milk Casein. The MO, NE, RB, and TE kits overestimated casein content at most incurred levels, with kit recoveries ranging from (lowest to highest incurred level) 303.5 to 139.2% (MO), from 374.1 to 162.8% (NE), from 191.8 to 82.8% (RB), and from 263.3 to 118.8% (TE) (Figure 1C). These kits are more accurate at the higher incurred casein levels. The measured values of the ES kit are questionable, however, because of the high background detected in the unfortified (0 ppm) level (Supporting Information, Supplemental Table 2C). Mean recoveries of the ES kit ranged from 193.8 to 36.7%, with casein content being underestimated at incurred levels above 2.88 ppm. Despite the inaccuracy at the lower incurred levels, all kits detected casein at the manufacturers' claimed LOQs using a dark chocolate matrix, but the quantitated levels were greatly overestimated.

Milk BLG. Mean measured values for BLG are shown in Figure 1D. Using the ES kit, measured BLG levels were consistent with all incurred levels, with mean kit recoveries ranging from (lowest to highest incurred level) 141.7 to 63.8%, and a mean recovery of 99.4% across all incurred levels. The MO kit greatly overestimated BLG at all levels with a mean recovery of 788.9% (Supporting Information, Supplemental Table 2D). Mean measured values using the TE kit were inconsistent with the incurred levels. For most incurred levels, the TE kit underestimated BLG. For some incurred levels, however, higher recoveries were also observed, particularly at the lowest incurred level, which raised the mean measured recovery of all fortified levels to 255.4%. These results indicate that although the TE and MO kits may provide the least accurate results when used for the detection of BLG, all kits detected BLG at the indicated LOQs claimed by the manufacturers.

Thus, the ability of each test kit to accurately quantify any one test allergen differed, and the accuracy varied depending on incurred level. Regression analysis revealed a functional linear relationship between the measured (M) and incurred protein levels (P) of untempered chocolate (Supporting Information, Supplemental Figure 1). The accuracy of each kit at any incurred level can be estimated by evaluating the slope of each plot. The closer the slope is to 1.0, the more accurate the kit, a slope = 1 (recovery = 100%) being the most accurate. For example, Figure 2 shows that the RB peanut kit underestimates the incurred peanut protein level (slope = 0.568, corresponding to a recovery 56.8% of the incurred protein level). On the basis of the slopes of the regression analyses (Table 2), the accuracy of each test kit for allergen detection in the untempered dark chocolate matrix from most to least accurate was as follows: for peanut protein, RB (0.57), NE (0.35), MO (0.17), TE (0.10), and ES (0.03); for egg protein, ES (1.02), TE (0.81), MO (2.04), NE (2.55), and RB (2.83); for milk (casein), TE (1.18), RB (0.83), MO (1.38), NE (1.62), and ES (0.37); for BLG, ES (0.64), TE (0.08), and MO (5.45). Accuracy should not be the

Table 2. Accuracy of Each Test Kit (and Corresponding Percent Recovery) from the Linear Regression Analysis Relating Measured Protein Level (M) to Incurred Protein Level (P) for Each Test Kit and Each Allergen in Untempered and Tempered Chocolate (All Intercepts Assumed to Be Zero)

kit manufacturer[a]	untempered chocolate	tempered chocolate
	Peanut	
R-Biopharm	0.57 (57%)	0.73 (73%)
Neogen	0.35 (35%)	0.35 (35%)
Morinaga	0.17 (17%)	0.11 (11%)
ELISA Systems	0.03 (3%)	0.03 (3%)
Tepnel	0.10 (10%)	0.29 (29%)
	Egg	
R-Biopharm	2.83 (283%)	2.55 (255%)
Neogen	2.55 (255%)	2.83 (283%)
Morinaga	2.04 (204%)	0..76 (76%)
ELISA Systems	1.02 (102%)	0.66 (66%)
Tepnel	0.81 (81%)	0.58 (58%)
	Milk (Casein)	
R-Biopharm	0.83 (83%)	0.02 (2%)
Neogen	1.62 (162%)	1.22 (122%)
Morinaga	1.38 (138%)	0.69 (69%)
ELISA Systems	0.37 (37%)	0.50 (50%)
Tepnel	1.18 (118%)	0.54 (54%)
	Milk (BLG)	
Morinaga	5.45 (545%)	3.57 (357%)
ELISA Systems	0.64 (64%)	0.44 (44%)
Tepnel	0.08 (8%)	0.02 (2%)

[a]Characteristics of each kit are listed in Supplemental Table 1 of the Supporting Information.

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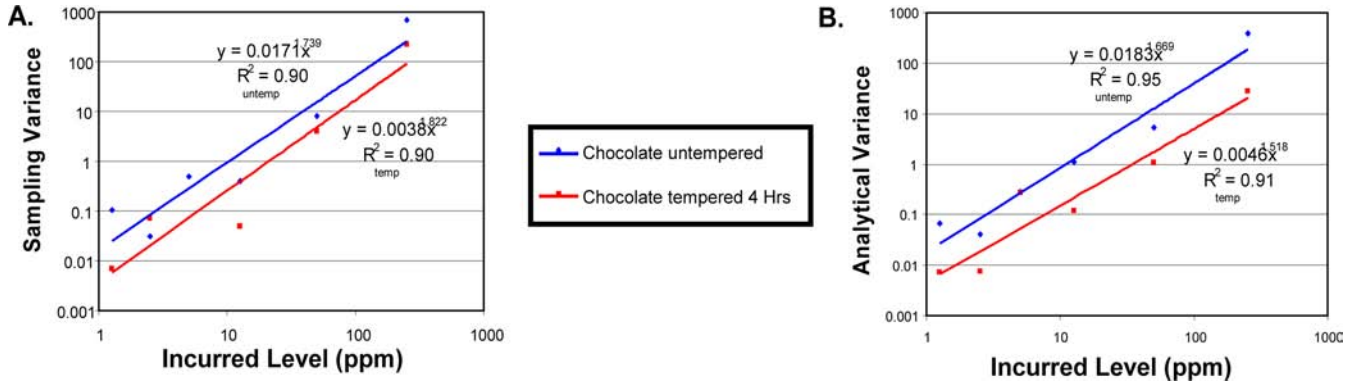

Figure 3. Regression analysis of (A) sampling and (B) analytical variance for the Neogen (NE) test kit used to measure peanut protein in untempered and tempered dark chocolate. Sampling and analytical variances increase with incurred allergen concentration.

only consideration, however, when a test method is chosen. It may be better to choose a kit that overestimates allergen levels (based on regression analysis) as opposed to a more accurate kit that underestimates the allergen content at the same incurred level to avoid a potential reaction at lower allergen levels that approach the LOQ of a particular kit.

Effect of Tempering on Allergen Detection Using Incurred Dark Chocolate Samples. Thermal processing can alter the physical and chemical properties of allergenic proteins, the interaction between the matrix components and the allergens, and recognition of the allergen by specific antibodies.[18] In addition to thermal processing effects, the components of the chocolate matrix can negatively affect the ability of the immunoassay to detect an allergen. Comparison of the mean measured value and the percent recovery for all test kits at all incurred levels in tempered chocolate are shown in Supplemental Table 2 of the Supporting Information. Allergen recovery from untempered and tempered dark chocolate, averaged across all levels, is shown in Figure 1.

Peanut. Mean recovery at all incurred levels of peanut for untempered and tempered chocolate, respectively, are as follows: ES (1.8%, 1.6%), MO (17.1%, 15.2%), NE (35.1%, 26.1%), RB (92.6%, 66.9%), and (11.8%, 18.8%). Tempering the dark chocolate did not greatly decrease the mean measured concentration or the percent recovery of peanut (Figure 1A). For most kits, however, the recovery was well below the incurred levels for both untempered and tempered samples, indicating that other factors affect peanut recovery more than the tempering process. Only the RB kits detected peanut protein close to the kit LOQ (2.5 ppm) in the tempered chocolate. At the incurred level approximating this LOQ (2.5 ppm), the mean measured level of peanut using the RB kit was 2.31 ppm, with a recovery of 91.5%.

Egg. The measured levels of egg protein were comparable for tempered and untempered chocolate for all test kits. Recovery varied among test kits, however, which is also observed when using these kits with other processed foods.[18,29] The NE and RB kits overestimated egg protein in tempered chocolate at all incurred levels with mean recoveries of 285.4 and 256.8%, respectively (Figure 1B). Although the mean measured recoveries of the ES, MO, and TE kits were 67.9% (ES), 88.6% (MO), and 81.0% (TE) in tempered chocolate, these kits performed better in this matrix compared to other processed matrices.[29] At the lowest incurred level in this study (1.2 ppm), mean measured protein and percent recoveries of the test kits were ES (0.9 ppm, 74.9%), MO (1.16 ppm, 96.8%), NE (4.09 ppm, 340.5%), RB (4.13 ppm, 343.8%), and (1.95 ppm, 162.7%). On the basis of these results, all of the kits were able to detect egg protein in the tempered dark chocolate matrix at or close to the LOQs claimed by the manufacturer, although some kits were more accurate than others.

Milk Casein and BLG. Tempering the dark chocolate reduced the casein and BLG recoveries using most kits, and in some cases the reduction was dramatic (Figure 1C,D and Supplemental Table 2C,D of the Supporting Information). Quantitative comparisons among casein kits and BLG kits for the tempered dark chocolate were difficult to conduct because the averaged recoveries across all incurred levels ranged from 0.5 to 99.7% for casein and from 15.5 to 410% for BLG. The NE kit was the most accurate, with a recovery of 99.7% across all incurred levels. Casein recovery using the TE kit was best at the incurred levels at or below the kit's LOQ, but the TE kit had a high background at the 0 ppm incurred level, calling these results into question. The ES and MO kits had mean recoveries of 53.0 and 80.0%, respectively, across all incurred casein levels. Tempering greatly reduced the recovery of the RB kit, for which there was little to no casein detected at any incurred level. Although tempering the chocolate significantly reduced the BLG levels measured by the MO kit, recovery was still 410.1% when averaged across all incurred levels. On the basis of these results, the NE, TE, and, possibly, the ES kits detected casein protein at their respective LOQs. Only the MO kit, however, could detect BLG at the manufacturer's claimed LOQ using the tempered chocolate matrix, and the quantitated level was greatly overestimated (i.e., inaccurate), similar to the untempered samples. Reductions in milk protein detection following processing are observed[14,21,37] and could be due to reduced solubility of the proteins being measured and/or a reduction in kit antibody binding to the proteins, which may have undergone conformational changes due to the tempering process.

The functional relationship between the measured and incurred values of tempered chocolate was determined for each kit and each allergen by linear regression analysis as described above for the unprocessed chocolate (Figure 2 for the RB kit for peanut, and for all kits in Supplemental Figure 1 of the Supporting Information). The accuracies of the test kits for allergen detection (based on the slopes of the regression analyses) are listed in Table 2. When the recovery of processed samples is evaluated, however, care must be taken in interpreting the results. Technically, recovery can be determined only if one assumes that the allergenic proteins

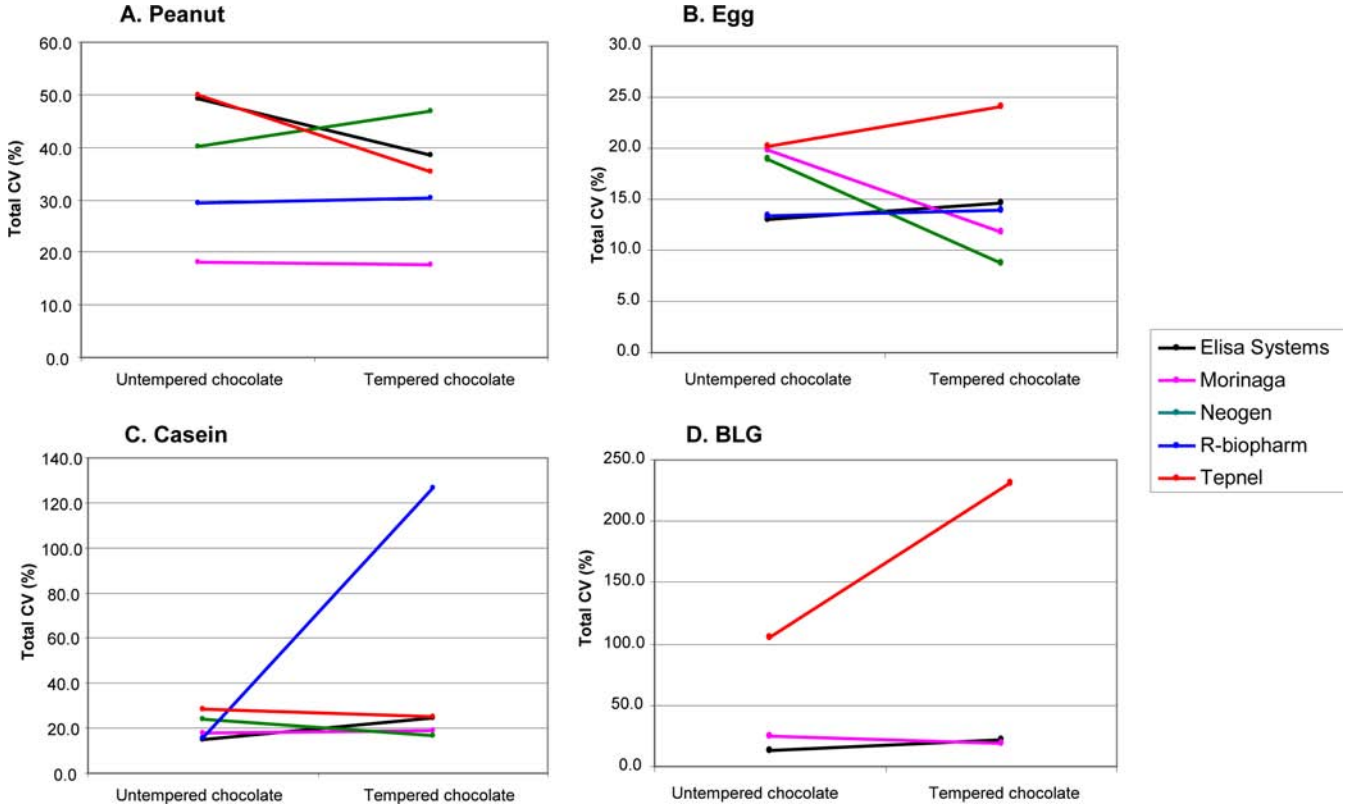

Figure 4. Total coefficient of variation (CV) averaged across all incurred levels for each kit and each allergen in untempered and tempered dark chocolate.

are not destroyed during processing. Whereas some proteins may be altered by processing conditions to the point of being undetectable by a specific antibody, they may retain their allergenicity. If the level of any allergenic protein was reduced by the tempering process, then recovery should be based on the new allergen level after tempering. In addition, some of the dark chocolate samples were intended to have incurred levels close to the LOQs of most of the test kits. In some cases, the measured allergen levels were below the manufacturers' claimed LOQ, but these data were included in the calculations of method performance for comparative purposes.

Sampling and Analytical Variation Associated with the Measurement of Peanut, Egg, and Milk Allergens in Untempered and Tempered Dark Chocolate. The calculated variance and CVs for peanut, egg, and milk allergens for the five test kits are shown in the Supporting Information, Supplemental Tables 3 and 4, respectively. The sampling variability represents differences in the measurements of the allergens among the four samples taken from each incurred level. The analytical variability represents the difference in measurements of the allergens among the four aliquots taken from each of the four sample extracts at each incurred level.[24] When plotted in full log, variance as a function of allergen concentration can be represented by the regression equation $y = ax^b$, where y is the variance, x is the incurred allergen concentration, and a and b are constants determined from the regression analysis (Figure 3 and Supplemental Figure 2 of the Supporting Information). The relationship between the variance and incurred concentration is similar for tempered samples for most kits when plotted on a full-log scale, and sampling and analytical variance of processed chocolate are comparable to those of untempered chocolate for most kits (Figure 3A for sampling variance, Figure 3B for analytical variance using test kit NE for peanut allergen, and for all kits in Supplemental Figure 2 of the Supporting Information). Sampling and analytical variance increased with an increase in the incurred level of all allergens using all test kits, with the exception of the TE kit for BLG, for which the sampling and analytical variance decreased with an increase in the incurred level using the untempered sample.

The analytical CVs were generally higher at the lower incurred levels for all test allergens (Supporting Information, Supplemental Table 4), but the analytical CVs of untempered chocolate were not significantly different from those of tempered chocolate when averaged across all incurred levels, with the exception of a few test kits. The sampling and analytical CVs were considerably higher for the tempered chocolate using the RB kit for casein at all incurred levels, whereas the analytical CVs were quite low using untempered samples. Sampling and analytical CVs of the TE kit for BLG were considerably higher than those of the other BLG kits using both tempered and untempered samples. When the RB kit for casein and the TE kit for BLG were excluded, the sampling and analytical CVs were comparable for all kits and all four allergens when tempered and untempered chocolate were compared. The mean sampling and analytical CVs for all allergens at all incurred levels are shown in Supplemental Table 4 of the Supporting Information. Analytical and sampling CVs were highest for peanut allergen detection for all test kits with the exception of the MO kit. Overall, kit sampling and analytical CVs were lowest for egg detection. In general, the sampling and analytical CVs contributed equally to total CV for most test kits and allergens in this matrix.

When averaged across all incurred levels for all kits (Figure 4; Supplemental Table 4E of the Supporting Information), total CVs were comparable for tempered and untempered incurred samples for peanut and egg determination. For casein and BLG, however, the total CVs were much higher using the tempered samples for certain kits, indicating that the tempering process interferes with the extraction efficiency of casein and BLG using these kits or alters the structure of these allergens so that allergen recognition by the kit antibodies is negatively affected. The heat sensitivity of casein and BLG was described previously.[14,21,28,37−38]

Quantitative comparisons between kits are challenging due to the different characteristics of the antibody-based kits, compounded by the heterogeneity of the analytes (multiple allergenic proteins) and differences in kit reporting units. Some useful comparisons can be made, however, particularly when the effect of processing on detection of a single allergen using an individual kit is evaluated. The peanut detection level using most kits was lower than the incurred level using the dark chocolate matrix, although peanut recovery was not reduced significantly after chocolate tempering, indicating that the tempering process had less influence on the detection of peanut allergen compared with the other test allergens. Results from tempered chocolate revealed that some kits were accurate in quantifying egg, casein, and BLG. The recovery of other kits was reduced, but not to the extent observed in other processed matrices.[14,18,21,29,37] This finding could be due to the differences in processing procedures, particularly the temperature at which the chocolate was tempered (46 °C). This temperature may not be high enough to cause structural changes in allergenic proteins that may occur in other matrices when higher processing temperatures are used. When averaged across all incurred levels, sampling and analytical CVs contributed equally to total CV for most test kits and allergens in this matrix. The process of tempering negatively affected sampling and analytical variability of some of the kits, particularly for the quantitation of milk casein and BLG. The key issues associated with method accuracy and precision can be addressed by using appropriate processed standards, by determining appropriate extraction procedures to be used with processed food and other difficult matrices, and by developing antibodies capable of recognizing allergenic proteins having epitopes that may be altered through various processing conditions.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental Tables 1−4 and Figures 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

BLG, β-lactoglobulin; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; LOQ, limit of quantitation.

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